

## THE MATERNAL POUCH AND DEVELOPMENT IN THE MARSUPIAL FROG *GASTROTHERCA RIOBAMBAE* (FOWLER)

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*Gastrotheca riobambae* (Fowler) is a common frog in the northern interandean valleys of Ecuador. In this genus, after the eggs leave the female's cloaca, the male places them inside a dorsal pouch in the body of the female (Walker, 1957; Matthews, 1957) where the embryos develop until the tadpole stage. Spannhof and Spannhof (1972), working with *Gastrotheca marsupiata*, found that incubation of embryos in the pouch lasts approximately five to six weeks, but the larvae may remain in the pouch for considerably longer periods, depending on environmental factors. After leaving the pouch, the tadpoles continue their development in water, where metamorphosis occurs in a few weeks. Hoogmoed (1967) has studied the mating and early development of *Gastrotheca marsupiata* and reviewed most of the existing literature. Additional observations on the embryonic incubation and larval development of *Gastrotheca marsupiata* have been reported by Spannhof and Spannhof (1972). Recent studies of the taxonomy and distribution of *Gastrotheca* in South America resulted in the conclusion that the populations of this genus in the vicinity of Quito are primarily *Gastrotheca riobambae* (Duellman and Fritts, 1972; Duellman, 1974). Although reports dealing with the pouch and reproductive activity of *Gastrotheca riobambae* are limited to the paper of Jones, Gerrard and Roth (1973), it is possible that some descriptions, such as those of Hoogmoed (1967), are actually based on *G. riobambae*, rather than *G. marsupiata*, since the animals were collected near Quito.

The maternal pouch of *Gastrotheca* is of uncommon interest as an adaptation to life on land, but surprisingly little is known about it. Experimental induction of pouch formation in juvenile females of *Gastrotheca riobambae* has been studied (Jones *et al.*, 1973), and aspects of the histology of the pouch of *Gastrotheca marsupiata* have been described (Spannhof and Spannhof, 1972); but the details of the histology and physiology of the pouch and the processes of embryonic development in the pouch have not yet been thoroughly studied. In this report we present the initial results of a study of reproduction in *Gastrotheca riobambae*, with particular emphasis on the structure of the pouch and embryos, maternal-embryonic relationships and changes in the pouch related to reproductive activity.

### MATERIALS AND METHODS

Adult specimens of *Gastrotheca riobambae* were collected in Quito, Ecuador, on the grounds of Pontificia Universidad Catolica del Ecuador, from other localities within the city, and near Machachi, a town located approximately 50 km south of Quito. The animals were brought into the laboratory and kept in a 1.50 × 0.40 ×

0.60 m terrarium. The terrarium was provided with vegetation and a tray containing pond water and several stones which gave supporting surfaces for the females at the time of tadpole hatching and emergence from the pouch. Both brown and green variants of the species were common at the collection sites and frogs of both colors were used for this study. Observations are based on a total of more than 50 frogs.

Ovulation was induced by the administration of 800 IU of human chorionic gonadotropin (Coriantin, Richter) into the coelomic cavity of the female. Males were stimulated to mate by the similar administration of 100 to 200 units. Mating occurred 24 hours or more after hormone administration. As stated, mating occurs on land, and, as they emerge from the cloaca, the eggs are moved into the pouch by the male. Sometimes only the female responded to the hormone treatment and in such cases the eggs released were not placed in the pouch but instead were deposited and left on the ground.

Pouch and embryos were fixed in Bouin's picro-formol, embedded in paraffin and cut into sections of 10  $\mu$ m thickness. Harris hematoxylin and alcoholic eosin yellow were used for routine staining. Whole mount permanent preparations of embryos were made by cutting the embryos from the yolk, fixing them in Bouin's and staining them with borax-carmin.

Eggs and embryos from the pouch were cultured in pond water or in several concentrations of amphibian Ringer's solution.

## RESULTS

### *General morphology of the pouch*

The pouch is a sac underlying the dorsal integument, but is essentially independent of it except at the aperture. Histologically, the pouch resembles amphibian integument, but differs significantly from it, particularly during reproduction. The pouch is absent in males and juvenile females, but is always present in the sexually mature female. The aperture of the pouch is triangular, with the apex of the triangle directed anteriorly (Fig. 1). The entrance to the pouch can be open or closed, depending on the stage of the reproductive cycle. In open pouches, the borders of the aperture are wide apart, giving a broad triangular or "U" shape (Fig. 1), whereas in the closed condition the borders of the aperture join in the midline of the body, giving a slit-like appearance (Fig. 2). The pouch aperture remains open after tadpole birth and throughout the growth of the next generation of oocytes in the ovary. Closure ordinarily occurs when the ovarian eggs have attained their full size just before the time of ovulation, but in females with large ovaries, closure can be induced experimentally in about twelve hours by injection of chorionic gonadotropin. The pouch remains closed during the time the embryos are being incubated, but even during this period it is easy to gain access to the pouch with a blunt probe or forceps for the removal of embryos. Under ordinary conditions, opening of the pouch occurs when the tadpoles are ready for hatching and release; we have observed, however, that handling of a frog with a closed pouch results in an immediate opening of the pouch. Following such an opening, if the animal is left undisturbed, the aperture closes again in about an hour.

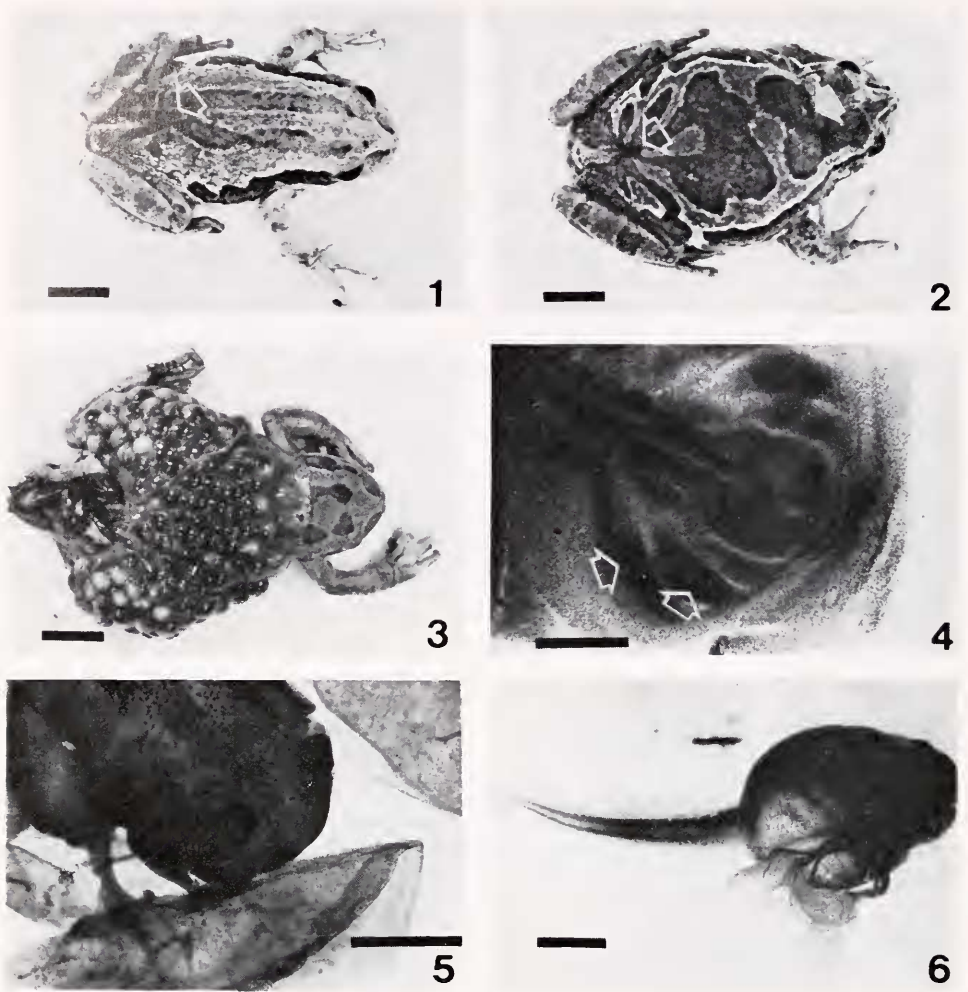


FIGURE 1. Female *Gastrotheca riobambae* before mating. The pouch aperture is open. Pouch aperture is triangular in shape, with the apex of the triangle (arrow) directed anteriorly. Bar represents 1 cm.

FIGURE 2. Female with developing embryos in the pouch. The pouch is considerably distended, with its anterior limit reaching almost to the head (solid arrow). The aperture of the pouch is closed and slit-like (open arrow). Bar represents 1 cm.

FIGURE 3. Female with developing embryos in the pouch. The dorsal integument has been removed to expose the intact distended pouch with the embryos inside. The pouch is almost transparent and fills the entire dorsal and lateral sides of the body. Bar represents 1 cm.

FIGURE 4. Early embryo removed from the pouch. The yolk was dissected and the embryo was prepared as a whole mount. The primordia of the bell gills are noticeable on both sides of the head (arrows). Bar represents 500  $\mu$ .

FIGURE 5. Advanced embryo removed from the pouch and jelly capsule (whole mount permanent preparation). The bell gills are large discs that in the living condition completely enveloped the embryo. Each gill is connected to the body by means of gill stalks (arrow). Bar represents 500  $\mu$ .

The pouch is attached to the integument and to the part of the body lying ventral to the pouch by thin lateral ligaments carrying blood vessels and nerves. In addition, there are long thread-like structures lying in the midline, carrying vessels and possibly nerves, which connect both the ventral and dorsal walls of the pouch with the area ventral to it. There is a sheet of muscle on the dorsal side, extending between the integument and the pouch. Although the pouch is a permanent structure, it varies considerably in size, depending upon the phase of reproduction. In nonpregnant females (*i.e.*, females without eggs in the pouch), the pouch extends approximately 1 cm anteriorly and laterally from the aperture. The pouch of the pregnant female becomes highly distended as the embryos develop, extending approximately 3 cm anteriorly from the aperture and bringing the limits of the fully-distended pouch anteriorly to the base of the head and laterally into the ventral portions of the body (Figs. 2 and 3). The distension of the pouch at this time is such that its walls become almost completely transparent (Fig. 3), in contrast to the essentially opaque condition of the nonpregnant pouch.

*Structure of the nonpregnant pouch and changes during reproduction*

The histological structure of the pouch before ovulation resembles that of amphibian integument. The pouch is lined with stratified squamous epithelium, which is closely associated with numerous simple alveolar mucous glands, usually with large lumina. Some glands have small lumina, and the cells of these glands are considerably swollen with accumulated secretory material. A few scattered serous glands are present. As compared with the skin, the pouch contains fewer mucous and serous glands, and its epithelium is thrown into numerous folds at this stage (Fig. 7). The pouch epithelium also appears less keratinized than the epithelium of the skin. Just below the basal membrane, chromatophores and sometimes small blood vessels are seen. The corium is thick, with scattered large blood vessels located deep within it. The part of the corium immediately below the basal membrane is loosely arranged and serves as a matrix for the glands, while the deeper layer contains dense fibers of connective tissue in parallel bundles, as well as some elastic fibers. A layer of muscle can sometimes be seen below the corium. The outer limit of the pouch is formed by a layer of mesothelium.

Only one female was studied for changes immediately following ovulation without mating, but in this animal the most noticeable difference between her pouch and pouches observed before ovulation was in the mucous glands. The lumina of these glands tended to be occluded by the enlarged secretory cells (Fig. 8), in contrast to their condition before ovulation. No change was detected in the number of glands. Folds were conspicuous in the epithelium.

Ovulation is usually followed by mating and filling of the pouch with eggs. The initial changes associated with incubation of the eggs appear to be the development of activity in the mucous glands and the thinning of the pouch. Enlargement of the pouch seems to be the result of the presence of eggs, presumably due to

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FIGURE 6. Living embryo of an advanced stage removed from the pouch and the jelly capsule. Before the jelly was removed, the gills completely enveloped the embryo, but removal resulted in collapse and shrinkage of the gills. The blood vessels within the gill stalks are prominent. Bar represents 2 mm.

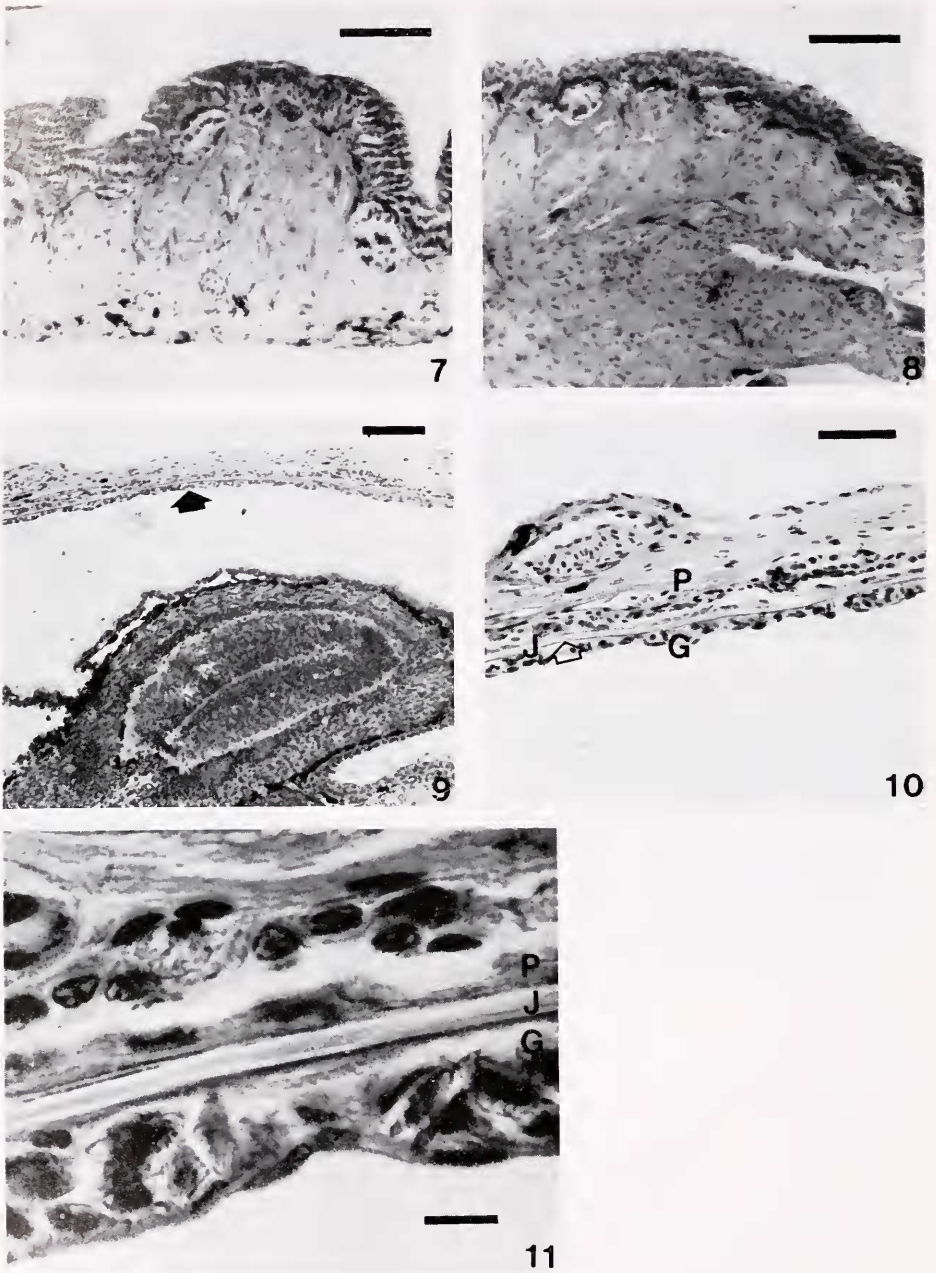


FIGURE 7. Cross section of the pouch before ovulation. Infoldings of the epithelium are prominent, and there are numerous simple alveolar glands with empty lumina. The epithelial layer is less keratinized than the integument, but the general appearance is similar. Bar represents 100  $\mu$ .

mechanical distension, since we observed that in one female that oviposited but did not mate, the pouch remained the same size fifteen days after egg laying.

Embryonic development in the pouch is accompanied by increased vascularization of the pouch lining and close association between pouch and egg jelly (Figs. 9, 10 and 11). As the pouch walls become thinner, numerous blood vessels invade the area of the basal membrane and therefore come to lie very close to the epithelium (Figs. 10 and 11). The mucous glands have large lumina and are rare, compared to the previous condition. Initially, the walls of the pouch are in simple contact with the jelly capsules of the embryos, but as development proceeds, each encapsulated embryo comes to be partially enclosed in a vascularized pocket of pouch tissue. The chambers are formed by the upper and lower walls of the pouch and by lateral projections from these walls around the embryos.

Embryonic incubation in the pouch ends with hatching and release of the tadpoles. Birth of the tadpoles is followed by shrinkage and thickening of the lining of the pouch. Immediately after birth, the egg pockets of the pouch are still present but are collapsed (Fig. 12); the pouch appears disorganized when compared with that of the pregnant female.

Regression of capillaries in the pouch follows the birth of the tadpoles and the projections which formed the embryonic chambers are withdrawn. Epithelial folds are prominent immediately following birth (Fig. 12), but reorganization of pouch tissue eliminates them either by invagination of the epithelium, followed by tissue reorganization within the layer of connective tissue, or by evagination and shedding. Extensive shedding of the epithelium was noticed some days after birth of the tadpoles. During reorganization, numerous compound alveolar glands with large lumina appear, located deep in the layer of connective tissue (Fig. 13).

In a female examined fifty-two days after birth of the tadpoles, the structure of the pouch appeared similar to that of the sexually mature female before ovulation, but epidermal folds were inconspicuous. Mucous glands were present but were of the simple alveolar type, with large lumina.

#### *Embryonic incubation and birth of tadpoles*

Early development, occurring inside the pouch, is essentially synchronous. Development is associated with an increase in size of the embryos, and the pouch becomes much expanded as development proceeds. Segmenting eggs taken from the pouch are approximately 3 mm in diameter and are covered by a thin but firm coat of jelly; they lack dark pigment and the yolk gives them a rather uniform

FIGURE 8. Cross section of the pouch immediately after ovulation. No eggs had been placed in the pouch. The pouch is similar to that prior to ovulation except for the mucous glands, whose cells appear to be enlarged with accumulated secretory material. Bar represents 100  $\mu$ .

FIGURE 9. Cross section of an embryo within the pouch. The embryo is surrounded by the fluid-filled space of the capsule. The bell gill (arrow) is flattened against the jelly, and the jelly lies against the vascularized tissue of the pouch. Detail is shown in the next two figures. Bar represents 100  $\mu$ .

FIGURE 10. Detail of association between bell gill (G), jelly (J), and pouch (P). Bar represents 50  $\mu$ .

FIGURE 11. Higher magnification of the gill-jelly-pouch relationship shown in Figure 10. Bar represents 10  $\mu$ .

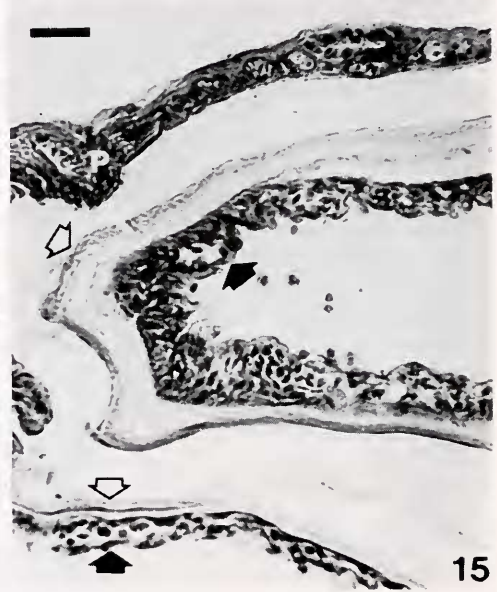
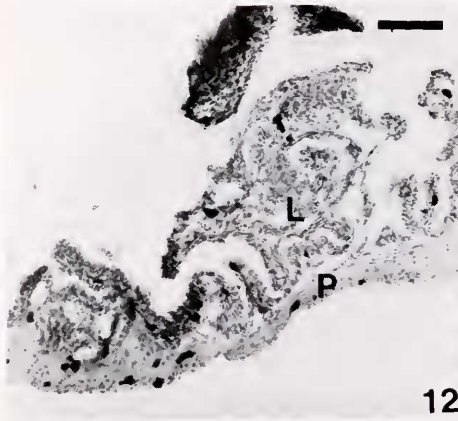


Figure 12. Pouch immediately following hatching and emergence of the tadpoles. The extensive folding and vascularization of the lining is evident. Bar represents  $100\ \mu$ .

FIGURE 13. Reorganizing pouch 27 days after emergence of the tadpoles. Note the presence of a compound alveolar gland. Bar represents  $100\ \mu$ .

FIGURES 14 AND 15. Cross section of two adjacent embryos within the pouch. Each embryo is associated with the epithelium of the pouch, but the jelly capsules of the two embryos were probably contiguous with each other before shrinkage was induced by fixation. P represents pouch; open arrows, jelly; solid arrows, gill. The bar in Figure 14 represents  $100\ \mu$ ; bar, Figure 15, represents  $50\ \mu$ .

yellowish-white color. The first cleavage divisions appear to divide the egg into four large blastomeres, but it is not known whether the cleavage furrows in the vegetal area are more than superficial. Development occurs at the animal end of the egg in a manner, described by Spannhofer and Spannhofer (1972), as similar to that

of a fish. The early development of *Gastrotheca riobambae* in the pouch is indeed different from that described for anurans such as *Xenopus laevis* or *Rana pipiens* (Nieuwkoop and Faber, 1967; Rugh, 1962). A unique developmental characteristic of *Gastrotheca* is the early appearance and subsequent marked growth of a pair of expanded gills, called "bell gills" (Noble, 1927). These gills originate from two paired masses of tissue, the gill primordia, located on either side of the head of the early embryo (Fig. 4). The functional gills of *Gastrotheca* originate from the fusion of these two primordia on each side. In the early gills the area of fusion of the two primordia, indicating their double origin, is easily distinguished. Later, the gills become small vascularized discs; still later, when fully developed (Figs. 5 and 6), they completely surround the embryo, with the right and left gills joining to form a highly vascular sac in intimate contact with the inner surface of the jelly coat (Figs. 9, 10, and 11). Each gill is connected to the embryo-proper by two long vascular stalks (Fig. 6); each stalk consists of one efferent vessel and one afferent vessel within a common membranous covering. Therefore, between each gill and the embryo there are four connecting blood vessels. Later in development, the gills appear to cease growth, thus at the time of hatching and birth, since the embryo has continued to grow, the gills no longer envelop the entire embryo. Although the gills appear simply to halt their growth, it is possible that they may actually decrease in size toward the later stages of development. At hatching, the vascularized discs of both gills protrude through the left operculum, but the gill stalks have been retracted and are no longer visible externally. Immediately after birth the gills are bright red; after a few minutes of contact with pond water, however, the red color disappears. Apparently the circulation through the gills stops soon after hatching, after which gill resorption occurs, taking approximately 24 hours. The histological constitution of the fully developed bell gill (Figs. 9, 10, 11, 14, and 15) appears as a thin epithelium containing numerous blood vessels associated with the basal membrane. The gills bear a general resemblance to lung tissue.

During the initial stages of embryonic development there is minimal association between pouch and embryos; young embryos can readily be extruded to the outside by slight external pressure on the pouch. As development continues, however, removal of embryos becomes somewhat more difficult. The increased difficulty of removal is due to the development of an intimate association between the gills of the embryo and the highly vascular lining of the pouch, with only the thin layer of jelly lying between the maternal and embryonic circulation (Figs. 9, 10, and 11).

Embryos may be distributed in the pouch in either a single or a double layer. In all cases, however, each embryo is in contact with pouch lining. Ordinarily, embryos do not seem to be completely enveloped by pouch tissue, although this might conceivably occur when there are few embryos in the pouch. Each embryo lies in an individual concavity of pouch tissue and in close contact with it, but the jelly of some embryos is contiguous (Figs. 14 and 15). In this situation, therefore, the vascular systems of adjacent embryos are separated from each other by their thin jelly capsules only.

The period of incubation within the pouch is somewhat variable. Under laboratory conditions we have observed a range of 103 to 120 days. Hatching usually



occurs in the pouch, followed by emergence aided by the mother. Just before emergence of the tadpoles, the female moves into water and rests her forelegs on a supporting surface. She then inserts the long toes of her hind legs into the pouch and, after a few seconds, one or two swimming tadpoles emerge. The female usually remains motionless for less than a minute before repeating the insertion process. In the laboratory, on occasion, not all the tadpoles from a female hatched on the same day, but rather over a period of two or more days.

The number of embryos per pouch varies considerably: in 11 females, the number ranged from 81 to 207, with a mean of  $125 \pm 10$  (s.e.). Unfertilized eggs or dead embryos in the pouch were rare, but when present, they had dried in the pouch and were expelled with the newly hatched tadpoles at the time of birth. Measurements of 42 newly hatched tadpoles from 8 females gave a snout to vent length of  $7.7 \pm 0.2$  mm, with a total length of  $20.2 \pm 0.4$  mm.

Segmenting eggs and very early embryos within the jelly do not survive in pond water but can be cultured in Ringer's solution for as long as seventeen days. Embryos cultured in Ringer's solution without aeration develop more slowly than those left in the pouch and appear to be deficient in blood formation. Aeration of the solution increases developmental rate and seems to improve blood production. Escape from the jelly does not occur in segmenting eggs and early embryos cultured *in vitro*, but advanced embryos hatch soon after being placed in pond water or Ringer's. Pouch embryos that have acquired the tadpole shape and have developed complete bell gills (Fig. 6) can be cultured either in pond water or Ringer's. In either medium, they soon hatch from the jelly and appear as very small free-living tadpoles. Resorption of the bell gills usually takes several days under these conditions.

As mentioned earlier, circulation through the bell gills apparently ceases soon after normal hatching, after which the gills are resorbed. If hatched tadpoles are kept in  $1.5\times$  Ringer's solution, however, the circulation and gills are maintained. Free-living tadpoles can be equally well maintained in pond water or in Ringer's solution.

## DISCUSSION

Reproductive adaptations toward a terrestrial mode of life have evolved in a number of amphibian groups. Among the anurans, *Nectophrynoides occidentalis* shows true viviparity (Angel and Lamotte, 1948). Others have not gone that far, but have abbreviated the aquatic dependence for embryonic development in many different ways (*cf.* Gallien, 1959; Noble, 1931). Members of the genus *Gastrotheca* are examples of the latter group. In this genus the requirement of an aqueous environment for embryonic development has been diminished or essentially eliminated, depending on the species. In *Gastrotheca ovifera*, young metamorphosed froglets are born from the pouch, while in *Gastrotheca marsupiata* and *Gastrotheca riobambae*, tadpoles are born (Matthews, 1957; Hoogmoed, 1967; Spannhof and Spannhof, 1972; this paper). In this latter case, the tadpoles metamorphose after only a few weeks of aquatic development. Mating in *Gastrotheca* occurs on land, in contrast to the more usual situation for anurans.

In evolution, the pouch of *Gastrotheca* may have originated as a fold of dorsal skin serving to protect the embryos in a terrestrial environment. One may suppose that the pouch could have served originally as a structure protecting the embryos against predation; at the same time, the surrounding tissue would also insure the embryos against desiccation. It may be that the pouch of *Gastrotheca* originated as shallow folds of skin similar to those bordering the egg mass carried on the back of the female *Fritziana* (see Trueb, 1974, for a discussion of the possible phyletic relationship of the South American pouched frogs). Development of the pouch (Jones *et al.*, 1973), which involves invagination of the integument, is in agreement with that idea.

Noble (1925) found that, in the Amphibia, reduction of functioning of the lungs is correlated with modifications of the skin which make it more effective in respiration. One of the modifications involves the penetration of capillaries from the dermis into a closer association with the epidermis. According to Noble, these capillaries lie just beneath the outermost epidermal layer. In a similar way, the vascularization lying close to the epithelium of the pouch in *Gastrotheca* during pregnancy, together with the general thinning of the pouch wall, appear to transform the pouch of pregnant females into an organ functioning in gaseous exchanges between mother and embryos. Noble (1925) mentioned the pouch as a specialized respiratory organ, and Spannhof and Spannhof (1972) observed not only the vascularization of the pouch in *G. marsupiata* but also the formation of egg chambers. We have seen what is apparently a similar situation in *G. riobambae*. It seems evident that the vascularization of the outgrowths forming the egg chambers enhances the capacity of the pouch for gaseous exchange. The maternal and embryonic circulatory systems are separated only by the thin (about 10  $\mu$ ) jelly capsule of the embryo, and this may be assumed to be of little hindrance to diffusion of gases.

In addition to the rather obvious respiratory function of the pouch, it is also possible that maternal-embryonic exchanges of water and/or other materials may occur between the closely apposed circulatory systems. Jones *et al.* (1973) support the idea of maternal-embryonic interchanges, but without reference to the nature of the materials supposedly exchanged and without specific evidence. Spannhof and Spannhof (1972), on the other hand, believe that probably no nutrient transfer occurs in the pouch, since they were able to maintain embryos and follow development for as long as 8 days outside the pouch. Evidence from the present study confirms the fact that embryos can be cultured outside the pouch for extended periods under certain conditions, but is of no help with regard to the matter of maternal-embryonic exchanges. Preliminary studies in our laboratory, however, reveal no appreciable change in the dry weight of embryos while in the pouch, suggesting that there is not a passage of nutrients from the mother into the embryo.

Hormonal factors are evidently important in pouch formation and function. Pouch formation has already been shown to be elicited by injection of estradiol into immature females (Jones *et al.*, 1973). Our observations suggest a hormonal role in opening and closing of the pouch aperture. It is possible that embryonic incubation, together with vascularization of the pouch and the formation of egg chambers, may also be under hormonal control. Preliminary experiments in our

laboratory, using ovariectomized pregnant females, give support to this possibility. Pouch function, at least insofar as opening and closing of the aperture is concerned, seems also to be subject to nervous control. This is manifest in the rapid opening of the pouch aperture when a pregnant female is handled.

The embryonic bell gills of *Gastrotheca*, along with the pouch, are clearly important adaptations in terrestrial reproduction, and it is worth noting that most frogs with incubating pouches develop this type of gill (Noble, 1931). The evidence of the present study indicates a double origin of the gills, as does the work of Spannhof and Spannhof (1972), who traced the origin of the blood vessels of the gills of *Gastrotheca marsupiatata* to the first and second aortic arches. The gills of *Gastrotheca* might have been multiple originally, similar to the situation in some species of *Cryptobatrachus* (Noble, 1927).

The study of reproduction in *Gastrotheca* is of considerable interest for comparative physiology, development and anatomy, as well as for the general problem of evolutionary adaptations to the terrestrial environment. In this case, the pouch and the pattern of development, along with associated hormonal and nervous control systems constitute remarkable modifications which make possible an extended period of independence from the aquatic environment. Further study of the details of reproduction in this species is in progress.

#### SUMMARY

The pouch of *Gastrotheca riobambae* (Fowler) serves as the location for development of the embryo up to the swimming tadpole stage. The pouch lies under the dorsal integument of the female and, in nonpregnant females, is similar to the integument. Pregnancy is accompanied by increased vascularization of the pouch. Blood capillaries from the corium become closely associated with the epithelial lining of the pouch, and vascularized outgrowths of the pouch partially envelop the embryos. Embryonic development in the pouch is characterized by the presence of peculiar gills, the highly vascularized "bell gills," which expand and flatten against the inner surface of the jelly capsule, forming an individual sac about each embryo and thus establishing a close relationship between embryonic and maternal circulatory systems. After birth of the tadpoles, the gills are soon resorbed and there is regression of the vascularization of the pouch. Reorganization of the tissue and shedding of the lining epithelium restore the pouch to the condition found in the nonpregnant female.

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